# Delayed maturation of an IL-12–producing dendritic cell subset explains the early Th2 bias in neonatal immunity

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**Primary neonatal T cell responses comprise both T helper (Th) cell subsets, but Th1 cells express high levels of interleukin 13 receptor α1 (IL-13Rα1), which heterodimerizes with IL-4Rα. During secondary antigen challenge, Th2-produced IL-4 triggers the apoptosis of Th1 cells via IL-4Rα/IL-13Rα1, thus explaining the Th2 bias in neonates. We show that neonates acquire the ability to overcome the Th2 bias and generate Th1 responses starting 6 d after birth. This transition was caused by the developmental maturation of $CD8\alpha^+CD4^-$ dendritic cells (DCs), which were minimal in number during the first few days of birth and produced low levels of IL-12. This lack of IL-12 sustained the expression of IL-13Rα1 on Th1 cells. By day 6 after birth, however, a significant number of $CD8\alpha^+CD4^-$ DCs accumulated in the spleen and produced IL-12, which triggered the down-regulation of IL-13Rα1 expression on Th1 cells, thus protecting them against IL-4–driven apoptosis.**

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Abbreviations used: Ag, antigen; BFA, Brefeldin A; FcγR, Fc γ receptor; Tg, transgenic.

Secondary exposure to antigen (Ag) in neonates usually leads to a lack of Th1 cells and a bias toward Th2 immunity (1–4). This unbalanced response may explain the susceptibility of neonates to microbial infections and allergic reactions (5). Strategies aimed at balancing neonatal Th1 and Th2 responses may thus facilitate the development of effective vaccines against infections (6) and treatments for allergies (7). Recently, we and others have discovered that both Th1 and Th2 cells develop during the primary response to Ag in neonates (8, 9). However, a second encounter with Ag gives rise to a strong Th2 response and a weak Th1 response.

Using OVA-specific TCR transgenic (Tg) DO11.10 T cells (10), we developed a neonate-to-neonate transfer system that allows us to track the fate of primary Th1 cells upon exposure to Ag (11). In this system, neonatal splenic T cells from 1-d-old DO11.10/scid mice are transferred into newborn (1-d-old) BALB/c mice, which are then challenged with Ag i.p. 2 wk later, primary T cell responses are evaluated in the spleen. Secondary responses are examined in this model by rechallenging the mice with Ag in CFA at 7 wk of age and analyzing T cell responses 10 d later. Using this model, we have shown that the lack of secondary Th1 responses stems from the apoptosis of Th1 cells driven by IL-4 produced by their Th2 counterparts (9). In this system, IL-4 does not signal through the conventional type I IL-4 receptor (IL-4Rα/$\gamma_c$) on Th1 cells (12). Rather, primary exposure to Ag induces the up-regulation of the IL-13Rα1 chain, which associates with IL-4Rα, giving rise to an IL-4Rα/IL-13Rα1 heteroreceptor, or type II receptor, through which both IL-4 and IL-13 can signal (13, 14). During in vitro recall or in vivo rechallenge with Ag, IL-4 from the Th2 cells utilizes the IL-4Rα/IL-13Rα1 heteroreceptor to drive apoptosis of Th1 cells, thus biasing the secondary immune response toward Th2 cells (9).

In this study, we investigated the mechanism underlying the up-regulation of IL-13Rα1

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in neonatal Th1 cells. Our data indicate that the delayed developmental maturation of a subset of DCs leads to a shortage in IL-12 production, allowing for up-regulation of IL-13Rα1 expression and its association with the IL-4Rα chain. Indeed, we show that the $CD8\alpha^+CD4^-$ DCs accumulate to a significant level by day 6 after birth, a time point at which expression of IL-13Rα1 decreases, leading to diminished Th1 cell apoptosis and the restoration of IFN-γ production by Th1 cells upon secondary Ag challenge. Neutralization of endogenous IL-12 on day 6 restored IL-13Rα1 expression and apoptosis of neonatal Th1 cells, thus inhibiting secondary IFN-γ responses, and supplying exogenous IL-12 during exposure to Ag at birth decreased IL-13Rα1 expression and inhibited apoptosis of primary Th1 cells, allowing the generation of secondary Th1 responses. Consistent with this, the transfer of wild-type adult DCs into newborn mice that had previously received neonatal DO11.10 T cells inhibited the expression of IL-13Rα1, leading to the survival of primary Th1 cells and the production of IFN-γ during secondary Ag challenge. In contrast, the transfer of IL-12–deficient DCs led to sustained IL-13Rα1 expression, Th1 cell apoptosis, and, thus, a lack of IFN-γ production during the secondary response. Finally, transfer of $IL\text{-}12^{+/+}$, but not IL-12–deficient, adult $CD8\alpha^+CD4^-$ DCs also inhibited IL-13Rα1 expression and Th1 cell apoptosis, and restored secondary IFN-γ production by Th1 cells. The effect of transferring $IL\text{-}12^{+/+}$ $CD8\alpha^+CD4^-$ DCs was reversed if IL-12 was neutralized at the time of Ag exposure.

## RESULTS

### Day 6 after birth represents a turning point for the expression of IL-13Rα1 and the development of neonatal Th1 immunity

Previously, we developed a neonate-to-neonate TCR Tg T cell transfer system that was adapted to overcome the limitations associated with the small size of the newborn mouse and made tracking T cell fate possible (8). In this system, neonatal (1-d-old) DO11.10 TCR Tg CD4 T cells that are specific for OVA 323–339 peptide (designated OVA) (10) are transferred into BALB/c newborns (1 d old), the recipients are exposed to Ag, and the T cells are traced with the TCR-OVA–specific anticlonotypic antibody KJ1-26 (15) and analyzed for response to Ag. Also, as Ag we used Ig-OVA, an Ig chimera expressing OVA peptide within the heavy chain variable region (9, 11), for the purpose of avoiding the use of adjuvant (4) and for optimization of peptide presentation by uptake through Fc γ receptors (FcγRs) (16). As indicated in Fig. 1, neonatal DO11.10 T cells transferred to 1-d-old BALB/c mice and exposed to Ig-OVA do not produce IFN-γ upon rechallenge with OVA peptide in CFA at adult stage (Fig. 1 A). When the transfer used 8-wk-old adult instead of neonatal T cells, secondary IFN-γ responses developed. The lack of a secondary IFN-γ

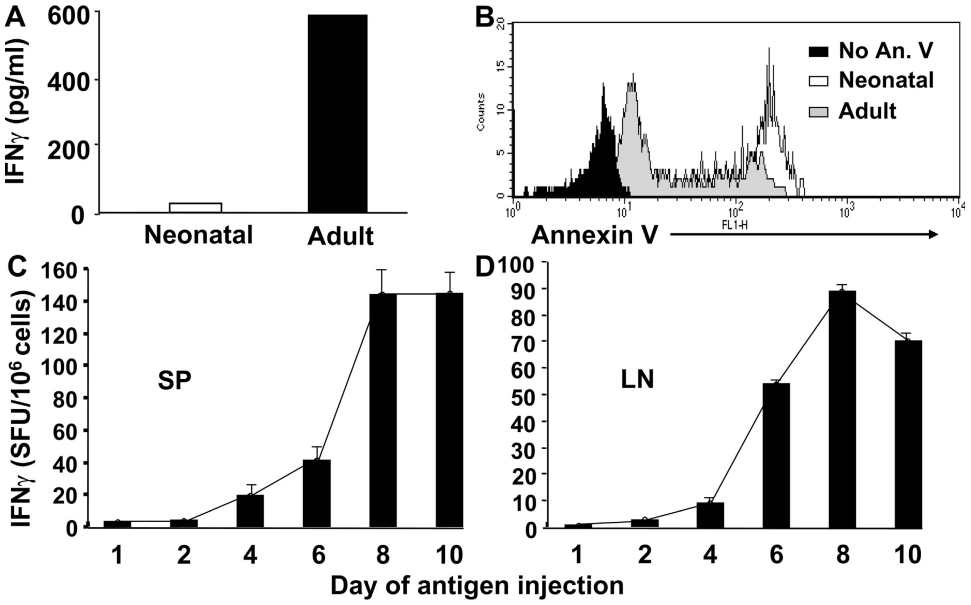


**Figure 1. Neonates acquire the ability to develop secondary Th1 responses when the primary encounter with Ag occurs on or beyond day 6 after birth.** Newborn BALB/c mice were given $3 \times 10^4$ neonatal or adult DO11.10 CD4 T cells and injected with 100 μg Ig-OVA in saline, and 2 wk later, the splenic cells ($10^6$ cells per well) were stimulated with 10 μM OVA peptide in vitro. (A) Production of IFN-γ was measured by ELISA. (B) Apoptosis of IFN-γ–producing $KJ1\text{-}26^+$ Th1 cells was evaluated by Annexin V staining. In B, 10 μg/ml BFA was added after 6 h of stimulation with OVA peptide, and the culture was continued for another 6 h to facilitate intracellular accumulation of IFN-γ. The cells were then labeled with KJ1-26 and Annexin V, and stained for intracellular IFN-γ. Histogram shows gating on $KJ1\text{-}26^+$/ $IFN\text{-}\gamma^+$ cells. (C and D) Newborn BALB/c mice were given $3 \times 10^4$ neonatal DO11.10 T cells within 24 h after birth and were injected with 60 μg/g Ig-OVA (to adjust for growth) on day 1, 2, 4, 6, 8, or 10 after T cell transfer. 2 mo later, the mice were challenged with 125 μg OVA peptide in CFA. 10 d later, the splenic ($10^6$ cells/well) and lymph node ($0.5 \times 10^6$ cells/well) cells were stimulated with 10 μM OVA peptide for 24 h, and production of IFN-γ was determined by ELISPOT. Each bar represents the mean ± SD of triplicate wells. The data are representative of two experiments.

response is because of the fact that neonatal Th1 cells undergo apoptosis upon reencounter with OVA peptide, as indicated by the substantial Annexin V staining relative to adult T cells (Fig. 1 B). However, if neonatal exposure to Ig-OVA occurs around day 6 after birth, the cells acquire the ability to develop secondary IFN-γ responses both in the spleen and lymph node upon rechallenge with OVA/CFA at the age of 7 wk (Fig. 1, C and D). Overall, neonates acquire the ability to develop Th1 secondary responses when the initial exposure to Ag occurs on or beyond day 6 after birth.

We have previously shown that exposure to Ig-OVA at the neonatal stage induces up-regulation of IL-13Rα1 on Th1 cells and that such a chain associates with IL-4Rα to form an IL-4Rα/IL-13Rα1 heteroreceptor (9) through which both IL-4 and IL-13 can signal (13, 14). Also, neonatal exposure to Ig-OVA induces both Th1 and Th2 cells in the primary response, and upon rechallenge with OVA peptide the Th2 cells produce IL-4 that uses the IL-4Rα/IL-13Rα1 heteroreceptor on Th1 cells to signal for their death (9). Because restoration of secondary IFN-γ responses could occur when the primary exposure to Ig-OVA takes place around day 6 after birth, we sought to test whether at that point in time exposure to Ig-OVA no longer sustains up-regulation of IL-13Rα1 expression. Accordingly, 1-d-old BALB/c mice that had received neonatal DO11.10 T cells were given Ig-OVA on day 1, 2, 4, 6, or 10 after T cell transfer, and 2 wk later their splenic Th1 cells were isolated on the basis of IFN-γ secretion and tested for IL-13Rα1 mRNA expression. As shown in Fig. 2 A, IL-13Rα1 mRNA was up-regulated when exposure to Ig-OVA took place on day 1, 2, or 4 after birth. However, a significant decrease occurred when the Ig-OVA was given on day 6 or 10 after birth. Densitometry analysis indicated that IL-13Rα1 mRNA went down from 260% of GADPH mRNA on day 2 to 10% on days 6 and 10, indicating an inability of the Th1 cells to up-regulate IL-13Rα1 mRNA (Fig. 2 B). The decrease in IL-13Rα1 mRNA on day 6 after birth translates into reduction of cell-surface IL-13Rα1 protein. Indeed, IL-13Rα1 protein expression went down by 50% (from 91 to 41%) when exposure to Ag was performed on day 6 instead of day 1 after birth (Fig. 2 C). Furthermore, Western blot analysis of Th1 T cell lysates indicated similar results, as Il-13Rα1 decreased by half upon Ag exposure on day 6 instead of day 1 after birth (Fig. 2 D). The control 1C5 CTLL-2 cells transfected with the *IL-13Rα1* gene (17) expressed IL-13Rα1 protein, whereas the untransfected wild-type CTLL cells did not, indicating that the rabbit anti–IL-13Rα1 antiserum is specific for IL-13Rα1 protein. Overall, IL-13Rα1 mRNA and protein expression decreased significantly when exposure to Ag was made on day 6 after birth.

When IL-13Rα1 mRNA expression from these Th1 cells during neonatal exposure to Ig-OVA was superimposed upon IFN-γ production in the secondary response, an inverse correlation between IL-13Rα1 mRNA and the secondary IFN-γ response was observed (Fig. 2 C). Indeed, when neonatal exposure to Ig-OVA was performed on day 6, the mice began to develop secondary IFN-γ responses when challenged with

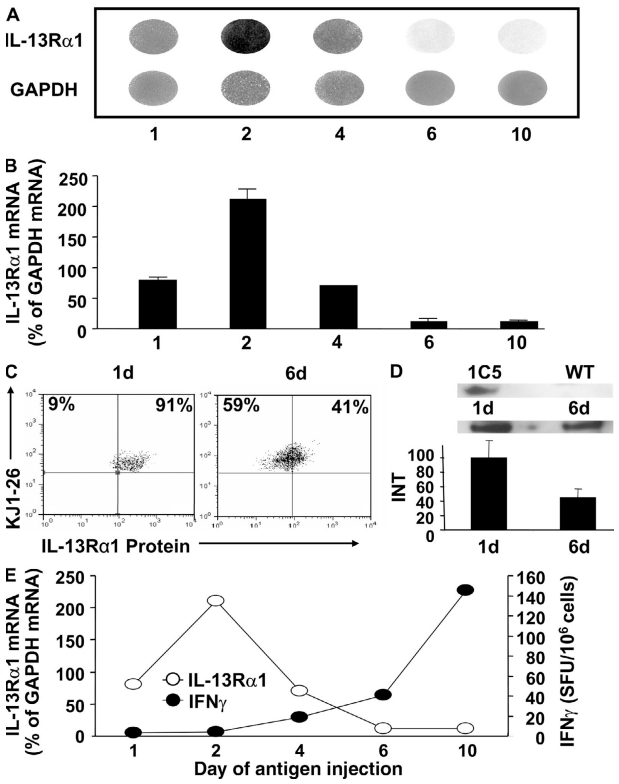


**Figure 2. Recovery of neonatal IFN-γ response coincides with down-regulation of IL-13Rα1 expression.** Newborn BALB/c mice were given 3 × $10^5$ purified neonatal CD4+ DO11.10 T cells and injected i.p. with 60 μg/g Ig-OVA in saline at day 1, 2, 4, 6, or 10 after birth, and 2 wk later, the splenic cells ($10^7$ cells/ml) were stimulated for 10 h with 10 μM OVA peptide. (A) Subsequently, Th1 cells were isolated, and RNA was extracted and used for analysis of *IL-13Rα1* gene expression as well as *GAPDH* control by spot-blot technology, as described in Materials and methods. (B) The intensity of radioactive spots was analyzed on a Molecular Imager FX using Quantity One software and presented as a ratio of *IL-13Rα1* to *GAPDH* after deduction of the background intensity obtained with pUC19 DNA negative control. The bars represent the mean ± SD of two experiments. (C and D) Newborn BALB/c mice were given 3 × $10^4$ neonatal CD4+ DO11.10 T cells and injected i.p. with 60 μg/g Ig-OVA in saline at day 1 or 6 after birth, and 2 wk later, the splenic cells ($10^6$ cells/ml) were stimulated for 10 h with 10 μM OVA peptide. In C, the cells were stained with KJ1-26 and rabbit anti–IL-13Rα1 antiserum (1:100 dilution), and Th1 cells were identified by staining for intracellular IFN-γ with anti–IFN-γ antibody. Expression of surface IL-13Rα1 was determined by flow cytometry on cells gated for KJ1-26 expression and intracellular IFN-γ production. In D, Th1 cells were purified as in A, lysed using NP-40 detergent, and run on a 10% acrylamide gel. Transfer was made onto a nitrocellulose membrane, and IL-13Rα1 protein was detected using rabbit anti–IL-13Rα1 antibodies (1:1,000 dilution). For control purposes, we used lysates from CTLL-2 cells transfected with a plasmid coding for cell-surface IL-13Rα1 (reference 17) or wild-type untransfected CTLL-2 cells. Band intensity was analyzed using the Molecular Imager FX, and the relative expression of IL-13Rα1 represents a percent ratio with 1 d as 100%. Each bar represents the mean ± SD of duplicate samples. (E) IL-13Rα1 mRNA expression measured by spot intensity from the experiments in A and B is illustrated together with the IFN-γ production presented in Fig. 1 C to correlate IL-13Rα1 down-regulation together with an increase in secondary IFN-γ production.

OVA peptide 2 mo later. Such IFN-γ responses were even higher when the neonatal exposure to Ig-OVA took place on day 10 after birth. Overall, day 6 represents a time point at which exposure to Ag no longer up-regulates IL-13Rα1, leading to the survival of IFN-γ–producing cells and the development of neonatal Th1 immunity.

**Exogenous IL-12 given during exposure to Ag on the day of birth reduces up-regulation of IL-13Rα1 on primary neonatal Th1 cells and restores IFN-γ secondary responses**

Previously, it has been shown that neonates demonstrate a poor ability to produce IL-12 (18), which justifies the lack of neonatal Th1 immunity because IL-12 is a key differentiation factor for Th1 cells. However, the primary response does indeed raise Th1 cells (8, 9), suggesting that the lack of neonatal secondary Th1 immunity is not due solely to defective differentiation. Given that IL-12 was shown to restore neonatal secondary splenic Th1 responses (4, 11), we thought that it may accomplish this effect by interfering with the up-regulation of IL-13Rα1 expression. To test this premise, newborn BALB/c mice were given neonatal DO11.10 T cells and were administered Ig-OVA together with rIL-12. 7 wk later, the animals were challenged with OVA peptide in CFA and their splenic secondary responses were analyzed. As seen in Fig. 3 A, the mice given IL-12 developed IFN-γ responses, whereas control animals not given IL-12 had no measurable IFN-γ responses. In line with these results is the observation that the apoptosis of Th1 cells from IL-12–recipient mice

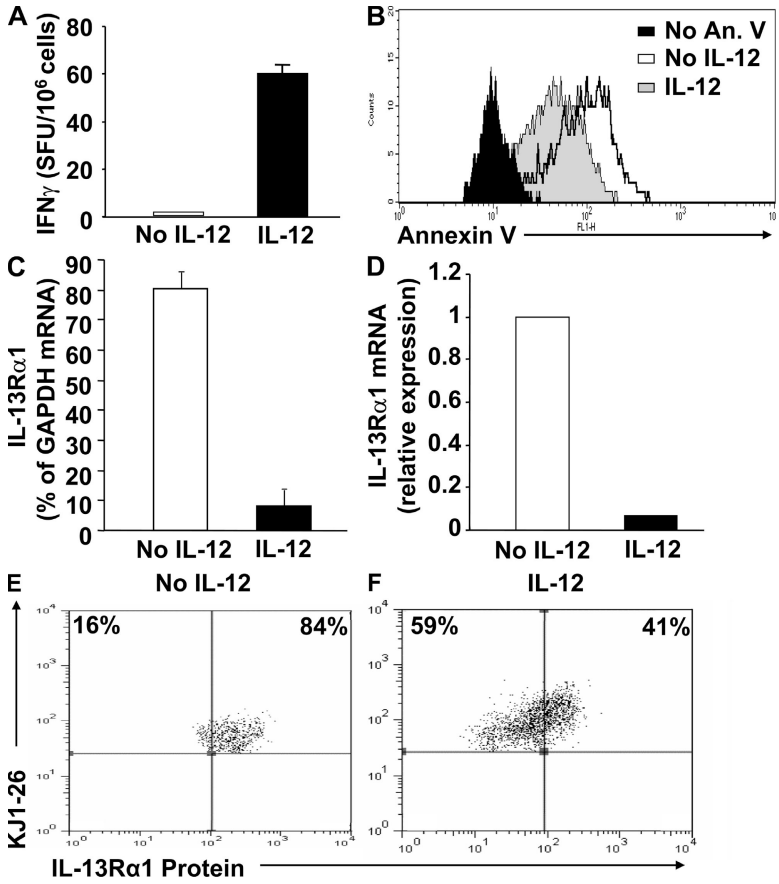


**Figure 3. Exogenous IL-12 inhibits developmental IL-13Rα1 up-regulation and restores secondary Th1 IFN-γ response in vivo.** Newborn BALB/c mice were given 3 × $10^4$ neonatal DO11.10 T cells within 24 h after birth. 1 d later, the recipient mice were given 100 μg Ig-OVA and 50 ng rIL-12. The mice were given additional IL-12 on days 2 and 3 after T cell transfer. 7 wk later, the mice were challenged with 125 μg OVA peptide in CFA, and 10 d after the challenge, the splenic cells ($10^6$ cells/well) were stimulated for 24 h with 10 μM OVA peptide. (A and B) Subsequently, IFN-γ was measured by ELISPOT (A) and apoptosis was evaluated using Annexin V staining of KJ1-26$^+$/IFN-γ$^+$ splenic cells (B). For Annexin V staining, 10 μg/ml BFA was added during the last 8 h of peptide stimulation. Each bar in A represents the mean ± SD of triplicate wells. (C and D) Mice that received neonatal DO11.10 T cells were given Ig-OVA and IL-12 as in A, and 2 wk later, Th1 cells were isolated and *IL-13Rα1* expression was assessed by spot blot (C) and real-time PCR (D). The real-time PCR used 200 ng RNA and the Absolute QRT-PCR SYBR kit to determine IL-13Rα1 mRNA. For the spot blot in C, each bar represents the mean ± SD of duplicate spots. For the real-time PCR, the bars represent the comparative threshold cycle ($C_T$). The value of the sample from mice not receiving IL-12 was set as 1. (E and F) Newborn BALB/c mice given 3 × $10^4$ neonatal CD4$^+$ DO11.10 T cells were exposed to Ig-OVA in the presence of IL-12 as in A and B, and 2 wk later, the splenic cells ($10^6$ cells/ml) were stimulated for 10 h with 10 μM OVA peptide. The cells were then stained with KJ1-26 and rabbit anti–IL-13Rα1 antiserum (1:100 dilution), and Th1 cells were identified by staining for intracellular IFN-γ with anti–IFN-γ antibody. Expression of surface IL-13Rα1 was determined by flow cytometry on cells gated for KJ1-26 expression and intracellular IFN-γ production.

was significantly reduced relative to control animals not given IL-12 (Fig. 3 B). When the animals were evaluated for IL-13Rα1 expression 2 wk after exposure to Ig-OVA/IL-12 but before any rechallenge with OVA/CFA, the primary Th1 cells had significantly reduced IL-13Rα1 mRNA relative to mice given Ig-OVA but no IL-12, as measured by spot blot (Fig. 3 C) and real-time PCR (Fig. 3 D). Again, when the cells were analyzed for IL-13Rα1 protein, the supply of exogenous IL-12 together with Ig-OVA reduced IL-13Rα1 protein expression from 84 to 41% when compared with cells from mice that did not receive IL-12. (Fig. 3, E and F). Overall, exogenous IL-12 was capable of down-regulating IL-13Rα1 on primary Th1 cells, which inhibited their apoptosis during rechallenge with OVA peptide and restored the secondary IFN-γ responses.

### Day 6 transition to Th1 responses is caused by an acquired ability of the neonate to produce IL-12 that down-regulates IL-13Rα1 and sustains the survival of IFN-γ–producing T cells

Because exogenous IL-12 was able to reduce IL-13Rα1 expression and rescue neonatal primary Th1 cells similar to Ag exposure on day 6, we postulated that the newborn acquires the ability to produce significant IL-12 on day 6, which results in IL-13Rα1 down-regulation and rescue of Th1 immunity. To test this premise, mice were given Ig-OVA and anti–IL-12 antibody on day 6 after birth, challenged with OVA peptide in CFA 2 mo later, and tested for IFN-γ production as well as Th1 apoptosis. Fig. 4 A shows that neutralization of IL-12 during exposure to Ig-OVA on day 6 reduces IFN-γ production to minimal levels comparable to those observed with day 1

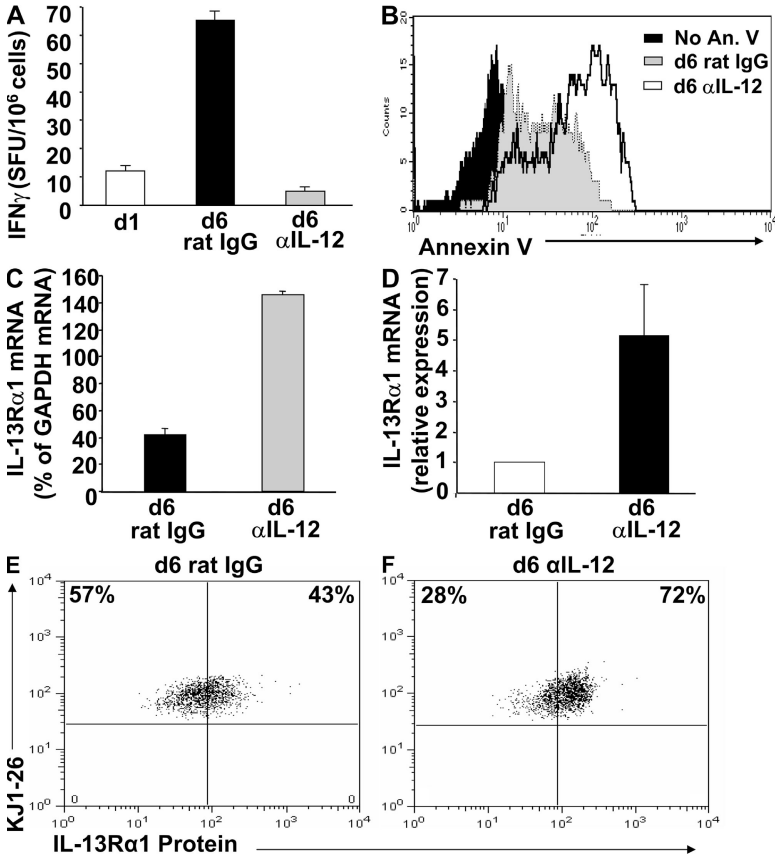


**Figure 4. Neutralization of IL-12 on day 6 after birth nullifies IFN-γ response through up-regulation of IL-13Rα1 and apoptosis of Th1 cells.** Newborn BALB/c mice were given 3 x $10^4$ CD4 neonatal DO11.10 T cells within 24 h after birth. On day 6, the hosts were given 60 μg/g Ig-OVA and 50 μg anti–IL-12 antibody per mouse or rat IgG. Another injection of anti–IL-12 antibody or rat IgG was given on days 7 and 8. A group of mice that received Ig-OVA on day 1 and no anti–IL-12 antibody at any time was included as a control. 7 wk later, all groups were challenged with 125 μg OVA peptide in CFA. 10 d later, the splenic cells ($10^6$ cells/well) were stimulated with 10 μM OVA peptide and assayed for (A) IFN-γ production by ELISPOT and (B) apoptosis by Annexin V staining, as in Fig. 3. Each bar represents the mean ± SD of triplicate wells. (C and D) Mice that received neonatal DO11.10 T cells were given Ig-OVA and anti–IL-12 antibody as in A, and 2 wk later, primary Th1 cells were isolated and IL-13Rα1 expression was assessed by spot blot (C) and real-time PCR (D), as described in Fig. 3. For the spot blot in C, each bar represents the mean ± SD of duplicate spots. For the real-time PCR, the bars represent the comparative threshold cycle ($C_T$). The value of the sample from mice that received rat IgG was set as 1. (E and F) Newborn BALB/c from A and B that received anti–IL-12 antibody or rat IgG control during exposure to Ig-OVA on day 6 were killed 2 wk later, and the splenic cells were stained with KJ1-26 and rabbit anti–IL-13Rα1 antiserum (1:100 dilution). The Th1 cells were identified by staining for intracellular IFN-γ with anti–IFN-γ antibody, and expression of surface IL-13Rα1 was determined by flow cytometry on cells gated for KJ1-26 expression and intracellular IFN-γ production.

exposure to Ag, whereas rat IgG–injected mice sustain significant IFN-γ responses. Also, apoptosis of Th1 cells was more substantial in the mice given anti–IL-12 antibody versus recipients of rat IgG (Fig. 4 B). These results indicate that the restoration of secondary Th1 responses upon delay of Ag exposure to day 6 after birth operates through the production of endogenous IL-12. Interestingly, IL-13Rα1 mRNA expression analyzed 2 wk after day 6 Ag exposure was significantly up-regulated in the mice given anti–IL-12 antibody versus those recipients of rat IgG instead (Fig. 4, C and D). Similar results were observed when the cells were analyzed for IL-13Rα1 protein expression (Fig. 4, E and F). Indeed, neutralization of IL-12 with ant–IL-12 antibody during day 6 exposure to Ig-OVA reduced IL-13Rα1 protein expression from 72 to 43% relative to cells from mice that received the control rat IgG instead of anti–IL-12 antibody. Overall, the findings suggest that the newborn mice acquire the ability to produce significant IL-12 by day 6 after birth that is sufficient for down-regulation of IL-13Rα1, survival of Th1 cells, and restoration of secondary IFN-γ responses.

### Enrichment with adult DCs inhibits IL-13Rα1 up-regulation on primary neonatal Th1 cells and restores secondary IFN-γ responses

IL-12 is produced by APCs, most significantly by DCs, during Ag presentation and direct T cell differentiation to Th1 (19). Most of the APCs in the neonate, however, are composed of macrophages and B cells, with a minimal number of DCs (11). It is thus possible that the lack of IL-12 in the neonates is caused by delayed developmental accumulation of DCs. To test this assumption, we enriched the neonates with splenic DCs from adult mice and tested them for rescue of primary neonatal Th1 cells from apoptosis and development of secondary Th1 IFN-γ responses. As can be seen in Fig. 5, binding of Annexin V to neonatal Th1 DO11.10 T cells decreased from 87% in the mice that did not receive DCs (Nil) to 12% in those that received DCs from IL-12–sufficient (IL-$12^{+/+}$) mice (IL-$12^{+/+}$ DCs; Fig. 5, A and B). Transfer of DCs from IL-12–deficient (IL-$12^{-/-}$) (20) instead of IL-$12^{+/+}$ mice did not reduce Th1 cell death, as binding of Annexin V was observed on 86% of the Th1 cells in comparison to 87% in the mice with no DC transfer (compare Fig. 5 C with Fig. 5 A). The primary Th1 cells from the IL-$12^{+/+}$ DC–enriched mice had reduced expression of IL-13Rα1 mRNA relative to T cells of mice that were transferred with IL-$12^{-/-}$ DCs or animals that did not receive DC transfer (Fig. 5 D). Furthermore, when the mice were challenged with OVA/CFA at 7 wk of age, those recipients of IL-$12^{+/+}$ DCs developed secondary IFN-γ responses, whereas animals that were not given DCs or received IL-$12^{-/-}$ DCs had minimal IFN-γ responses (Fig. 5 E). The conclusion to be drawn from these experiments is that enrichment with IL-$12^{+/+}$ DCs is similar to treatment with exogenous IL-12, which reduces IL-13Rα1 expression by Th1 cells, leading to the inhibition of apoptosis and restoration of secondary IFN-γ responses.

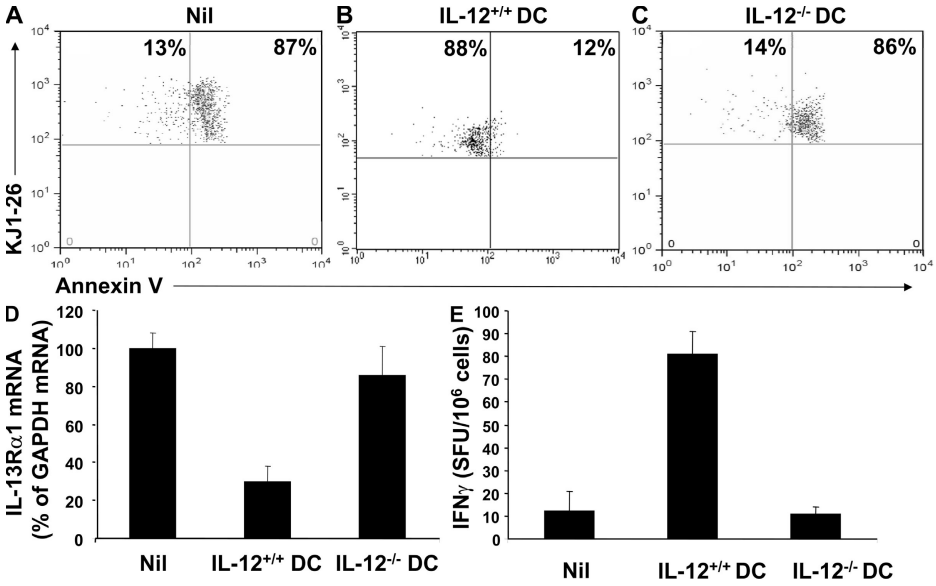


**Figure 5. Transfer of adult IL-$12^{+/+}$ but not IL-$12^{-/-}$ DCs into newborn mice down-regulates IL-13Rα1 expression, inhibits apoptosis, and restores secondary Th1 IFN-γ responses.** Newborn (1-d-old) BALB/c mice were given $3 \times 10^4$ neonatal DO11.10 T cells without (Nil) or with $2 \times 10^5$ splenic DCs from adult wild-type (IL-$12^{+/+}$ DCs) or IL-12–deficient (IL-$12^{-/-}$ DCs) BALB/c mice (reference 20) and were injected 1 d later with 100 μg Ig-OVA. After 7 wk, the mice were challenged with 125 μg OVA peptide in CFA, and 10 d later, the splenic cells ($10^6$ cells/well) were stimulated with 10 μM OVA peptide for 24 h. (A–C) Apoptosis of KJ1-26$^+$/IFN-γ$^+$ Th1 cells was evaluated by staining with Annexin V, as described in Fig. 3. (D) Groups of mice were killed 2 wk after T cell/DC transfer and exposure to Ig-OVA, and primary Th1 cells were isolated using IFN-γ secretion kit, as described in Materials and methods. RNA was then extracted and IL-13Rα1 expression was determined by spot blot, as in Fig. 2. Because IL-13Rα1 mRNA parallels with protein expression (see Figs. 2, 3, and 4), all follow-up experiments will use mRNA analysis on separated Th1 cells. (E) IFN-γ production was measured using ELISPOT assay. Each bar represents the mean ± SD of duplicate spots.

### Delayed developmental accumulation of the CD8α⁺CD4⁻ subset of DCs in the neonates leads to minimal IL-12 production, significant up-regulation of IL-13Rα1 expression, death of primary Th1 cells, and lack of secondary IFN-γ responses

Because DCs were able to reduce IL-13Rα1 expression, inhibit apoptosis of primary Th1 cells, and restore secondary IFN-γ responses, we sought to determine whether delayed developmental accumulation of a specific subset of DCs is responsible for the up-regulation of IL-13Rα1 by virtue of minimal IL-12 production leading to the death of primary Th1 cells and a lack of secondary IFN-γ responses. The splenic CD11c$^+$ DCs comprise both CD8α$^+$ and CD8α$^-$ DCs (21). Also, some of the CD8α$^-$ DCs can express CD4, and as such are further divided into CD8α$^-$CD4$^+$ and CD8α$^-$CD4$^-$ DCs. Initially, we determined the kinetics of accumulation of various subsets of DCs in the splenic neonate between the day of birth and day 10 of age. As indicated in Fig. 6, most splenic CD11c$^+$ DCs were negative for CD8α and CD4. However, by day 4 the number of CD8α$^-$CD4$^+$ DCs rose to 11.2% among the total CD11c$^+$ DCs, whereas CD8α$^+$CD4$^-$ DCs made up 3.3% of total CD11c$^+$ cells. By day 6, both subsets reached a number that was more or less similar to days 8 and 10, which ranged between 13 and 16% for CD8α$^-$CD4$^+$ and between 3.8 and 4.3% for CD8α$^+$CD4$^-$ DCs (Fig. 6). These numbers are much lower than those observed in adult mice (40.4% for CD8α$^-$CD4$^+$ and 16% for CD8α$^+$CD4$^-$ DCs), indicating that developmental accumulation is not complete by day 10. In fact, CD8α$^+$CD4$^+$ DCs, which make up a very small fraction of total adult CD11c$^+$ DCs, remain insignificant at day 10 of age. Because day 6 was a turning point for the development of Th1 secondary immunity and the CD8α$^-$CD4$^+$ and CD8α$^+$CD4$^-$ DC subsets have undergone a significant increase in number between birth and day 6, we tested both populations for reduction of IL-13Rα1 up-regulation, inhibition of apoptosis of Th1 cells, and restoration of secondary IFN-γ responses. Accordingly, newborn BALB/c mice were transferred with 1-d-old DO11.10 T cells with the CD8α$^-$CD4$^+$ or CD8α$^+$CD4$^-$ adult DC subset from either IL-12$^{+/+}$ or IL-12$^{-/-}$ mice and were exposed to Ig-OVA. 2 wk later, Th1 cells were isolated and IL-13Rα1 mRNA expression on primary neonatal Th1 cells was evaluated by spot blot. Fig. 7 A shows that up-regulation of IL-13Rα1 expression occurred when the mice received no DC transfer or transfer with IL-12$^{+/+}$ CD8α$^-$CD4$^+$ DCs. However, up-regulation of IL-13Rα1 expression was minimal when the animals were transferred with IL-12$^{+/+}$ CD8α$^+$CD4$^-$ DCs (Fig. 7 A). Transfer of IL-12$^{-/-}$ CD8α$^+$CD4$^-$ DCs did not reduce IL-13Rα1 mRNA expression on Th1 cells (Fig. 7 A). Moreover, apoptosis of Th1 cells was reduced significantly with the transfer of IL-12$^{+/+}$ CD8α$^+$CD4$^-$ DCs relative to IL-12$^{+/+}$ CD8α$^-$CD4$^+$ or no DC transfer (Fig. 7, C–E). Indeed, 83 and 87% of primary Th1 cells bind Annexin V when the transfer used no DCs or IL-12$^{+/+}$ CD8α$^-$CD4$^+$ DCs, respectively (Fig. 7, C and D). Only 45% of primary Th1 cells bound Annexin V when the transfer was made with IL-12$^{+/+}$ CD8α$^+$CD4$^-$ DCs (Fig. 7 E). Inhibition of T cell death by CD8$^+$CD4$^-$ DC transfer is dependent on IL-12. This statement emanates from the observation that IL-12$^{-/-}$ CD8$^+$CD4$^-$ DC transfer does not inhibit the apoptosis of Th1 cells (Fig. 7 F), but a supply of exogenous IL-12 together with the IL-12$^{-/-}$ CD8$^+$CD4$^-$ DC transfer restores the reduction of Annexin V binding to Th1 cells (Fig. 7 H). Finally, secondary IFN-γ responses did occur with the IL-12$^{+/+}$ CD8α$^+$CD4$^-$ DCs, but not with the IL-12$^{-/-}$ CD8α$^+$CD4$^-$ or even the

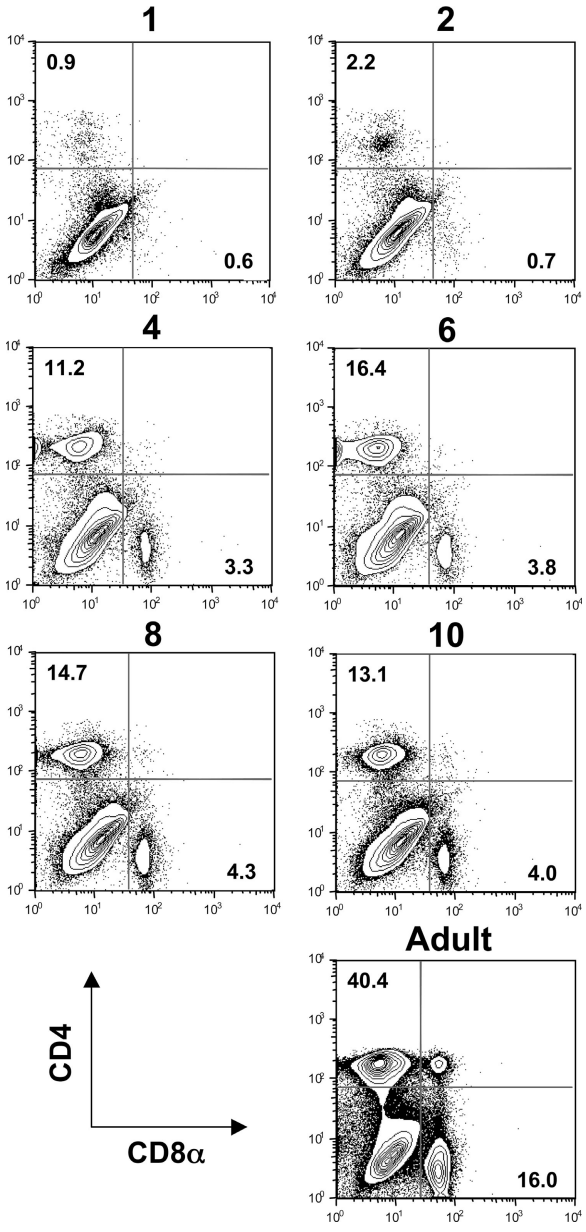


**Figure 6. Developmental accumulation of CD8α⁺CD4⁻ and CD8α⁻CD4⁺ DCs occurs at day 6 after birth.** Splenic cells ($10^6$ cells/tube) from 1-, 2-, 4-, 6-, 8-, and 10-d-old BALB/c neonates were stained with anti-CD11c, anti-CD4, and anti-CD8α antibodies, fixed with 2% formaldehyde, and analyzed by FACS. Expression of CD4 and CD8α was analyzed on cells gated on CD11c. Splenic cells from adult (8-wk-old) BALB/c mice were included as control. Percentages of cells are shown.

IL-12$^{+/+}$ CD8α$^-$CD4$^+$ DC transfer (Fig. 7 B). Supply of exogenous IL-12 with the IL-12$^{-/-}$ CD8α$^+$CD4$^-$ DCs restored IFN-γ secondary responses. Furthermore, neutralization of IL-12 during IL-12$^{+/+}$ CD8α$^+$CD4$^-$ DC transfer by injection of anti–IL-12 antibody restored apoptosis of primary Th1 cells (Fig. 7 B). These results indicate that CD8α$^+$CD4$^-$ DCs provide sufficient IL-12 needed for down-regulation of IL-13Rα1, inhibition of apoptosis, and restoration of secondary Th1 responses.

To determine whether the limited availability of IL-12 on the day of birth is caused by delayed accumulation of CD8α$^+$CD4$^-$ DCs or by an inability of these APCs to produce IL-12, we isolated neonatal CD11c$^+$ DCs at different time points after birth, stimulated the cells with the DC stimulator CpG dinucleotide, and tested for IL-12 production. As indicated in Fig. 8, neonatal DCs gain the ability to produce IL-12 progressively over time and reach a maximum at adult age (Fig. 8 A). However, these results do not distinguish between an increase in the number of CD8α$^+$CD4$^-$ DCs or the gain of ability to produce IL-12 cytokine. Given that isolation of the DC subsets at early neonatal age is not feasible because of low frequency, we opted to purify total CD11c$^+$ DCs, stimulate them with CpG, and test for intracellular IL-12 on gated DC subsets. Fig. 8 B shows that on day 1 after birth most of the splenic DCs are CD8α$^-$CD4$^-$, with almost no CD8α$^+$CD4$^-$ or CD8$^-$CD4$^+$ DC subsets. By day 6, however, both CD8α$^+$CD4$^-$ and CD8$^-$CD4$^+$ DC subsets reached significant frequency, as was shown in Fig. 6. When tested for intracellular IL-12, 26% of the CD8α$^-$CD4$^-$ double-negative and most frequent subset produced IL-12 on day 1 after birth, and 6 d later 84% of the cells displayed IL-12 expression (Fig. 8 C). This points to a developmental influence on these DCs to produce IL-12 cytokine. As for the CD8α$^+$CD4$^-$ DCs, like the CD8α$^-$CD4$^+$ DCs, there was no detectable IL-12 on day 1 but the majority of the cells available on day 6 produced IL-12 (84 and 97%, respectively; Fig. 8, D and E). These results indicate that the limited availability of IL-12 on the day of birth is caused by delayed accumulation of the DC subsets in the spleen, together with a progressive ability to produce IL-12 cytokine.

## DISCUSSION

Neonatal exposure to Ag leads to secondary responses that are usually biased toward Th2 upon rechallenge with Ag later (1–4).

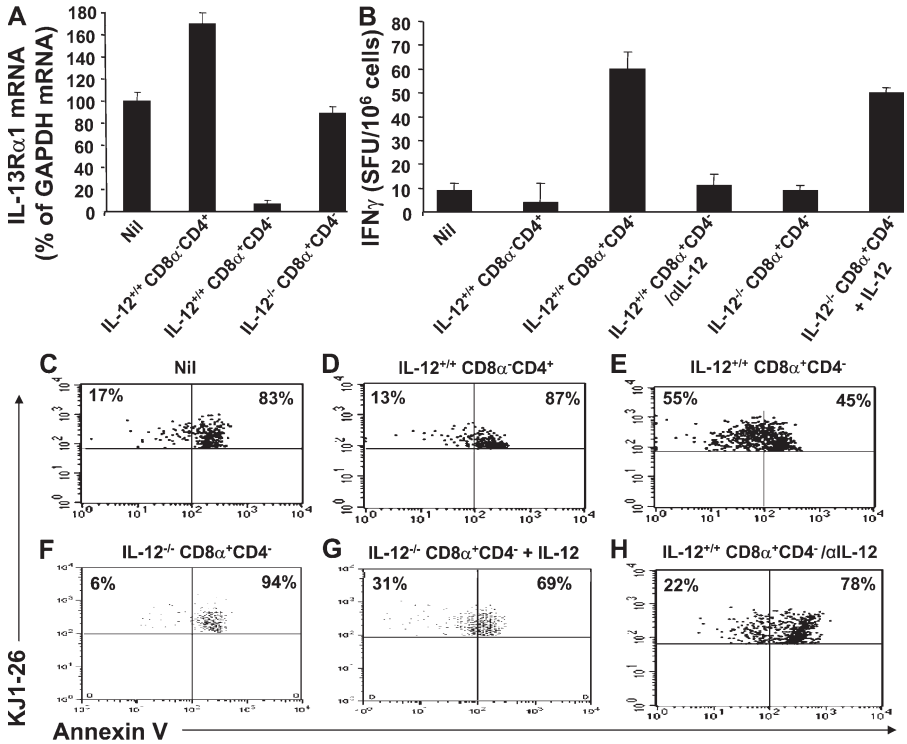


**Figure 7. The CD8α$^+$CD4$^-$ but not the CD8α$^-$CD4$^+$ DC subset inhibits up-regulation of IL-13Rα1, prevents apoptosis, and restores neonatal IFN-γ responses.** Newborn BALB/c mice were given neonatal DO11.10 T cells without (Nil) or with $10^5$ CD8α$^+$CD4$^-$ or CD8α$^-$CD4$^+$ DCs from adult IL-12$^{+/+}$ or IL-12$^{-/-}$ BALB/c mice and were injected 24 h later with 100 μg Ig-OVA. (A) The mice were killed within 2 wk, primary Th1 cells were isolated on the basis of IFN-γ secretion, and IL-13Rα1 expression was evaluated by spot blot. Each bar represents the mean ± SD of duplicate spots. (B–H) 7 wk after exposure to Ig-OVA the mice were challenged with 125 μg OVA peptide in CFA. 10 d later, the splenic cells ($10^6$ cells/well) were stimulated for 24 h with 10 μM OVA peptide, and production of IFN-γ was measured by ELISPOT (B) and apoptosis was evaluated by Annexin V staining of KJ1-26$^+$/IFN-γ$^+$ splenic cells (C–H). For neutralization of endogenous IL-12 where indicated (IL-12$^{+/+}$ CD8α$^+$CD4$^-$/anti–IL-12), the mice were given i.p. 50 μg anti–IL-12 antibody (clone C17.8) on days 1, 2, and 3 after neonatal transfer of DO11.10 T cells and DCs. For stimulation with OVA peptide in the presence of rIL-12 where indicated (IL-12$^{-/-}$CD8α$^+$CD4$^-$ + IL-12), the culture was supplemented with 2 ng/ml rIL-12. Each bar in B represents the mean ± SD of triplicate wells.

Recent investigations indicated that both Th1 and Th2 cells develop in the neonatal primary response, but secondary encounter with Ag leads to death of the Th1 cells by apoptosis, hence the bias toward Th2 cells (9). The death of Th1 cells stems from an up-regulation of the IL-13Rα1 chain during neonatal exposure to Ag (9). Indeed, it was shown that the IL-13Rα1 chain associates with IL-4Rα to form an IL-4Rα/IL-13Rα1 heteroreceptor through which both IL-4 and IL-13 cytokines can signal (13, 14). During rechallenge with Ag, IL-4 from the Th2 cells utilizes the IL-4Rα/IL-13Rα1 heteroreceptor and induces the death of Th1 cells (9). In this paper, we show that day 6 after birth is a turning point at which exposure to Ag no longer results in secondary responses that are biased toward Th2 and secondary Th1 IFN-γ responses emerge (Fig. 1). These observations bode well with findings indicating that exposure to Ag at day 7 but not at day 3 become effective in inducing Th1 responses (22). Interestingly, day 6 after birth also represents a turning point for the expression of IL-13Rα1, as exposure to Ag at this age no longer up-regulates IL-13Rα1 mRNA or protein expression on Th1 cells (Fig. 2). We then set up experiments and investigated the mechanism underlying the restoration of neonatal Th1 secondary responses by day 6 Ag exposure. We have previously shown that IL-12 can rescue splenic Th1 cells and restore IFN-γ production (4, 11). Given that the neonate displays an inability to produce significant IL-12 (19, 23), we thought that exposure to Ag within an environment where IL-12 is limited may be responsible for up-regulation of IL-13Rα1 and the consequent death of Th1 cells. This assumption proved correct, as exogenous IL-12 given during Ag exposure on day 1 reduced IL-13Rα1 mRNA and protein expression on primary Th1 cells, inhibited their apoptosis, and restored secondary IFN-γ responses (Fig. 3). Moreover, it was shown that the administration of anti–IL-12 antibody during Ag exposure on day 6 after birth nullifies restoration of secondary IFN-γ responses, and this operates through the restitution of IL-13Rα1 up-regulation and apoptosis of Th1 cells (Fig. 4).

Given that DCs are the main producers of IL-12 and that the neonatal environment is composed of lower numbers of DCs relative to other APCs (11), it is possible that the inability of the neonatal environment to provide significant IL-12 is related to the minimal number of Ag-presenting DCs available at day 1 after birth. In fact, when newborn mice were enriched with adult IL-12$^{+/+}$ splenic DCs, up-regulation of IL-13Rα1 on primary Th1 cells was minimal, apoptosis was significantly reduced, and secondary Th1 IFN-γ responses were restored (Fig. 5). IL-12$^{-/-}$ DCs could not inhibit IL-13Rα1 up-regulation or restore Th1 IFN-γ responses. These observations, together with the day 6–acquired neonatal transition to secondary Th1 responses, suggest that developmental accumulation of DCs could be responsible for the bias of neonatal immunity toward Th2. Given that distinct subsets of DCs can perform different functions (24–27), delayed developmental accumulation of a specific subset of CD8α$^{+/-}$CD4$^{+/-}$ DCs over time could orchestrate the Th2 bias of neonatal immunity. Analysis of DC ontogeny in the neonate mouse indicated that although CD11c$^{+}$ CD8α$^{-}$CD4$^{-}$ DCs were present at a significant number on the day of birth, the CD8α$^{+}$CD4$^{-}$ and CD8α$^{-}$CD4$^{+}$ subsets began to accumulate in the spleen by day 4 and reached a significant percentage by day 6 (Fig. 6). A small population of CD8α$^{+}$CD4$^{+}$ DCs that is present in adult mice was not detected by day 10 after birth. Thus, these observations suggested that it is the CD8α$^{+}$CD4$^{-}$ and/or the CD8α$^{-}$CD4$^{+}$ subset that might be responsible for the acquired ability of the neonate to develop secondary Th1

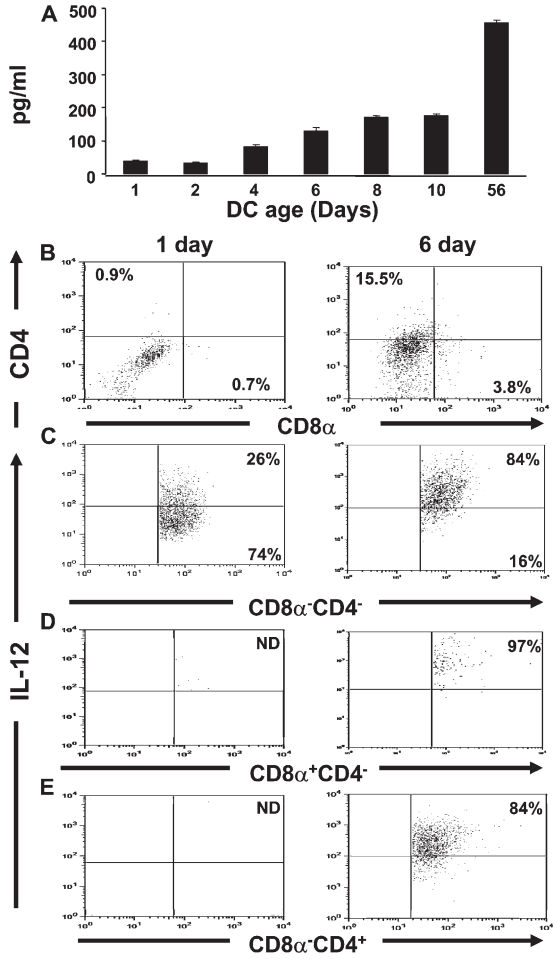


**Figure 8. The limited availability of IL-12 during neonatal exposure to Ag is related to delayed accumulation in the spleen of the presenting CD8α$^{+}$CD4$^{-}$ DC subset.** Newborn BALB/c mice were killed at the indicated day after birth, and splenic CD11c$^{+}$ DCs were isolated on anti-CD11c microbeads. (A) The DCs (100 × 10$^{3}$ cells per well) were stimulated with 6 µg/ml CpG, and 24 h later, IL-12 was measured by ELISA. Each bar represents the mean ± SD of triplicate wells. (B–E) 1- and 6-d-old DCs (100 × 10$^{3}$ cells per well) were purified from the spleens of 45 newborns, and 10$^{6}$ CD11c$^{+}$ cells were stimulated with 6 µg/ml CpG for 10 h, after which BFA was added and the culture was continued for 4 h to facilitate intracellular cytokine accumulation. Subsequently, the cells were stained with anti-CD11c, anti-CD4, and anti-CD8 antibodies and permeabilized with 2% saponin. Intracellular IL-12 was detected by staining with anti–IL-12 p70 antibody. B shows the frequency of the subsets, and C–E illustrates flow cytometry plots of intracellular IL-12 on the indicated subset. ND, not detected.

responses when exposure to Ag is performed on day 6 after birth. In fact, when the $CD8\alpha^+CD4^-$, but not the $CD8\alpha^-CD4^+$, subset was transferred to newborns on the day of birth during exposure to Ag, the primary Th1 cells displayed significantly reduced IL-13Rα1 expression, resisted apoptosis, and developed secondary IFN-γ responses when the animals were rechallenged with OVA as adults (Fig. 7). Transfer of $IL\text{-}12^{-/-}$ $CD8\alpha^+CD4^-$ DCs was not able to overturn IL-13Rα1 up-regulation or restore IFN-γ secondary Th1 responses, indicating that the subset presents Ag and produces the IL-12 needed to counter IL-13Rα1 up-regulation. Moreover, investigation of the frequency of the DC subsets indicated that a minimal number of the presenting $CD8\alpha^+CD4^-$ DC subset is present in the spleen on the day of birth. At best, these cells could produce IL-12 at a limited rate, like the $CD8\alpha^-CD4^-$ double-negative DC subset (Fig. 8), but this would be insufficient to counter IL-13Rα1 up-regulation. By day 6, the frequency of the $CD8\alpha^+CD4^-$ DC subset increased because of significant accumulation of this subset (Fig. 8). In addition, most of the cells produced IL-12, leading to inhibition of IL-13Rα1 up-regulation, diminished apoptosis of primary Th1 cells, and development of secondary Th1 responses.

Collectively, these observations indicate that delayed developmental maturation of a subset of DCs operates the Th2 bias of neonatal immunity. In particular, our data suggest that minimal production of IL-12 in early neonatal life because of delayed accumulation of the $CD8\alpha^+CD4^-$ DC subset leads to up-regulation of IL-13Rα1 on primary Th1 cells. This chain associates with IL-4Rα and forms an IL-4Rα/IL-13Rα1 heteroreceptor (13, 14). IL-4 from the Th2 cells used this receptor to drive apoptosis of Th1 cells, leading to a lack of secondary Th1 immunity, and hence the bias to Th2 cells (9). These observations agree with previous papers showing a delayed developmental accumulation of DCs (28, 29). What this study adds is that a specific subset is responsible for Ag presentation and IL-12 production, and it ties the regulation of IL-13Rα1 to such IL-12 and the developmental maturation of the $CD8\alpha^+CD4^-$ DC subset during the neonatal period. Also, we demonstrate that sufficient accumulation of the $CD8\alpha^+CD4^-$ DC subset by day 6 after birth represents a turning point to down-regulate IL-13Rα1 and sustain the transition for Th1 responses. These previously unrecognized findings highlight the mechanism underlying the bias of neonatal immunity toward Th2 cells and provide useful information for the development of pediatric vaccines and reagents against allergies in infants and children (30).

## MATERIALS AND METHODS

### Mice

BALB/c ($H\text{-}2^d$) mice were purchased from Harlan Sprague Dawley and were used as hosts for the adoptive transfer of T cells. DO11.10/scid Tg mice expressing a TCR specific for OVA peptide (10) were used as T cell donors. $IL\text{-}12^{-/-}$ mice in which the *IL-12p35* gene was knocked out (20) were obtained from the Jackson Laboratory and were used as a source for DC transfer. New Zealand white rabbits were purchased from Myrtle's Rabbitry. All animals were maintained in the animal facility for the duration of the experiments. Experimental procedures performed on these animals were conducted according to the guidelines of the University of Missouri Columbia Animal Care and Use Committee.

### Ag's

OVA peptide (SQAVHAAHAEINEAGR) encompasses aa residues 323–339 of OVA and is immunogenic in BALB/c ($H\text{-}2^d$) mice. Ig-W, a BALB/c IgG2b Ig molecule generated by transfection of the 91A3 antiarsonate antibody heavy and light chains into the non–Ig-secreting myeloma B cell line SP2/0, was previously described (4). Ig-OVA, expressing OVA peptide within the heavy chain variable region of Ig-W, was also previously described (11). ODN1826 or CpG oligonucleotide was purchased from InvivoGen.

### Generation of rabbit anti–IL-13Rα1 antibodies

**Expression and purification of IL-13Rα1 protein.** The plasmid pQE30–IL-13Rα1 (31), a gift from A. Gessner (Institut für Klinische Mikrobiologie, Immunologie und Hygiene, Erlangen, Germany), was digested with SphI and XhoI to liberate a cDNA that encodes the extracellular domain of IL-13Rα1 (aa 27–339) with an N-terminal His tag. This fragment was ligated into pFastBac plasmid (Invitrogen) to generate pFastBac–IL-13Rα1, which was then used to generate baculovirus and to express rmIL-13Rα1 in $Sf^9$ cells, according to the manufacturer's instructions. IL-13Rα1 protein was purified using Ni-NTA agarose beads from QIAGEN, as previously described (32).

**Rabbit immunization with IL-13Rα1 protein.** A New Zealand white rabbit was immunized with 50 μg rIL-13Rα1 in CFA/PBS, as previously described (33). A subsequent immunization with 50 μg rIL-13Rα1 in IFA/PBS was given monthly for 3 mo.

### Adoptive T cell transfer

Splenic cells from 1-day-old DO11.10/scid mice containing the equivalent of $30 \times 10^3$ DO11.10 T cells were transferred into 1-day-old BALB/c mice by i.v. injection through the facial vein using a 30-gauge needle. To obtain four experimental newborn BALB/c hosts, 12 DO11.10/scid neonatal mice were used. For the transfer of adult T cells into newborn mice, DO11.10 T cells were purified from the spleens of adult DO11.10/scid mice with anti-CD4 antibody–coupled magnetic beads (Miltenyi Biotec) before injection into the host. In this case, two to three adult DO11.10/scid mice are needed to generate four neonatal BALB/c hosts. When the analysis requires separation of Th1 from Th2 cells, the transfer used $300 \times 10^3$ purified rather unseparated neonatal DO11.10/scid T cells. In this case, 60 newborn DO11.10/scid mice are needed to generate four BALB/c hosts. This 10-fold increase in T cell transfer was required for the isolation of sufficient numbers of T cells for RNA extraction and spot blot and real-time PCR analysis.

### Detection of apoptosis by staining with Annexin V

For cell-surface staining, the splenic cells from BALB/c hosts that received neonatal DO11.10/scid T cells and Ig-OVA were incubated for 20 min at 4°C with 5 μg/ml 2.4G2 mAb to block FcγRs on the cell surface before staining. The cells were then stained with the anti-TCR OVA clonotypic mAb KJ1-26 (mouse IgG2a) and FITC–Annexin V (BD Biosciences). Cytokine secretion was blocked by the addition of 10 μg/ml Brefeldin A (BFA; Epicentre), and the cells were fixed with 2% formaldehyde, permeabilized with 2% saponin (Sigma-Aldrich) in PBS buffer for 10 min at room temperature, and incubated with PE–anti–mouse IFN-γ. Isotype-matched controls were included in all experiments. All data were collected using a flow cytometer (FACScan; Becton Dickinson) and were analyzed with CellQuest software (Becton Dickinson) and Flowjo software (Tree Star, Inc.).

### Separation of Th1 cells

The separation of neonatal Th1 from Th2 cells requires an increased frequency of primary T lymphocytes. To overcome this technical difficulty, the transfer was performed with purified neonatal DO11.10/scid T lymphocytes instead of total splenic cells, and the number of transferred cells was increased from 30 to $300 \times 10^3$ cells per newborn BALB/c host. Consequently, each T cell subset

isolation experiment used four neonatal BALB/c hosts that required cells from 60 newborn DO11.10/scid mice. Accordingly, each BALB/c newborn was given $300 \times 10^3$ $CD4^+$-DO11.10 T cells purified from the spleens of neonatal (1-day-old) DO11.10/scid mice, and the hosts were injected i.p. with a saline solution containing 100 μg Ig-OVA. 2 wk later, the splenic T cells were stimulated for 10 h with 10 μM OVA peptide, and Th1 cells were separated on the basis of cytokine secretion (IFN-γ), as previously described (34), using separation kits (Miltenyi Biotec). In brief, after peptide stimulation the spleen cells were incubated with mouse IFN-γ catch reagent for 45 min, and the cell surface–captured cytokine was used to attach PE-conjugated anticytokine mAb by incubation with PE–anti–IFN-γ for 10 min on ice. Anti-PE–coupled microbeads were added, and the cells were incubated for 15 min at 4°C. Subsequently, IFN-γ–producing cells were separated by MACS (Miltenyi Biotec). Usually, $10^8$ splenic cells are used to obtain from 0.5 to $10^6$ Th1 cells.

### Detection of a subset of DCs

For cell-surface staining, the splenic cells ($10^6$ cells/ml) from different ages (1, 2, 4, 6, 8, and 10 d old) of BALB/c neonates were incubated for 20 min at 4°C with 5 μg/ml 2.4G2 mAb to block FcγRs on the cell surface and were stained with anti-CD11c for 30 min. Subsequently, the cells were stained with FITC-labeled anti-CD8α and PE-labeled anti-CD4 (BD Biosciences), and fixed with 2% formaldehyde.

### Isolation of DCs

For the isolation of bulk splenic DCs, spleens from adult BALB/c or $IL\text{-}12^{-/-}$ mice were collected and purified according to the standard collagenase/differential adherence method (35). In brief, the spleen was disrupted in a collagenase solution, and isolated DCs were floated on a dense BSA gradient. Subsequently, the cells were allowed to adhere to Petri dishes for 90 min at 37°C, washed, and incubated overnight at 37°C and 7% $CO_2$. The next day, floating DCs were harvested and used for transfer experiments. In some experiments, adult DCs were purified using anti-CD11c mAb–coupled microbeads (Miltenyi Biotec). Isolation of splenic DCs from neonates used 45 newborns for each time point and was performed using anti-CD11c mAb coupled to microbeads, as described.

For the isolation of DC subsets, the bulk DCs were incubated with anti-CD8α mAb–coupled microbeads (Miltenyi Biotec) and were separated into $CD8\alpha^+$ and $CD8\alpha^-$ populations by MACS. For preparation of $CD8\alpha^+CD4^-$ and $CD8\alpha^-CD4^+$DC subsets, the $CD8\alpha^-$ fraction was labeled with anti-CD4 mAb–coupled microbeads and separated by MACS. Each fraction was assessed for purity, and no population was used if contamination was >5%.

### Treatment with rIL-12 and anti–IL-12 antibodies

In vivo injection of rIL-12 (PeproTech) used 50 ng of cytokine in PBS injected i.p., together with Ig-OVA, within 24 h after T cell transfer. The mice were given two more injections (50 ng in PBS) 48 and 72 h later. Control groups were injected with PBS without cytokine. For neutralization of IL-12, we used C17.8 anti–IL-12 mAb (BD Biosciences), which was injected i.p. Rat IgG was used as a control.

### Cytokine ELISA and ELISPOT

**Measurement of cytokines by ELISA.** Splenic cells containing both T cells and APCs were incubated with Ag in 96-well round-bottom plates for 24 h. IFN-γ production was measured by ELISA using anticytokine antibodies according to BD Bioscience's instructions.

**Measurement of IFN-γ by ELISPOT.** Detection of IFN-γ by ELISPOT was performed as previously described (4, 9). In brief, HA-Multiscreen plates (Millipore) were coated with capture antibody, and free sites were saturated with DMEM culture media containing 10% fetal calf serum. Subsequently, $10^6$ splenic cells were added and the culture was stimulated with OVA peptide with or without blocking antibody. 1 μg/ml of biotinylated anti–IFN-γ antibody was added, and bound antibody was revealed with avidin-peroxidase. Spots were counted using Immunospot software (Cellular Technology Ltd.).

### Spot blot for detection of *IL-13Rα1* expression

Because of the small number of isolated Th1 cells, RNA purification yields small amounts insufficient to perform Northern blot analysis. We developed a spot-blot technology to overcome these limitations and performed *IL-13Rα1* analysis by both spot blot and real-time PCR. Accordingly, a 1-kb *IL-13Rα1* DNA fragment was digested from pCEP4IL-13Rα1 plasmid with BamH1 and purified by electroelution from a 1% agarose gel. 1 μl Tris buffer containing 5 ng IL-13Rα1, pUC19 or *GAPDH* DNA was deposited within marked circular spots on a nylon transfer membrane (Magnacharge; Osmonics). Duplicate spots were made for each sample. The membrane was dried for 30 min at 60°C, and the DNA was denatured in 0.5 N NaOH and neutralized with 0.5 M Tris buffer. The membrane was then used for detection of IL-13Rα1 mRNA. Total RNA was extracted from $0.5\text{–}1 \times 10^6$ purified Th1 cells using TRIzol reagent (Life Technologies). 5 μg RNA was used in an RT reaction together with α-[$^{32}$P]dCTP (GE Healthcare) to generate labeled cDNA probes by using the Ampolabeling-LRP kit (SuperArray Inc.). The specific primers for the amplification of target genes were purchased from SuperArray Inc. Subsequently, the labeled cDNA probes were hybridized to the membrane in a 0.75-ml hybridization buffer for 24 h at 60°C. The intensity of radioactive spots was analyzed on a Molecular Imager FX (Bio-Rad Laboratories) using Quantity One software. Results were expressed as arbitrary units estimated as follows: (mean spot density of sample − mean spot density of background)/(mean spot density of GAPDH − mean spot density of background). The background represents the mean radioactive intensity obtained from pUC19 DNA spots included in the membrane.

### Real-time PCR

RT and DNA amplification were performed according to a one-step protocol using 200 ng of total RNA and an Absolute MAX QRT-PCR SYBR Mix (ABgene), according to the manufacturer's instructions. Expression of IL-13Rα1 chain mRNA was assessed in neonatal Th1 cells. β-actin was included to serve as a normalizer. The oligonucleotides used as specific primers were as follows: sense, (IL-13Rα1) 5′-GCACAGAGTATAGGTAAGGAGCAA-3′ and (β-actin) 5′-AGAGGGAAATCGTGCGTGAC-3′; and antisense, (IL-13Rα1) 5′-ACAAAGACTGGAATGGTGAGTAAC-3′ and (β-actin) 5′-CAATAGTGATGACCTGGCCGT-3′. Real-time PCR was performed on a Smart Cycler (Cepheid), and the results were analyzed by the comparative $C_T$ method described by the Smart Cycler software.

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